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Microdermatology: Cell Surface in the Interaction of Microbes with the External World†

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For many microbiologists, including the present author, the greatest attraction of the field of microbiology is our ability to analyze at the molecular level the physiological and ecological responses of microbes to the environment, thanks to the unicellular nature of most of these organisms. Thus, in microbiology there is hardly the separation seen in zoology and botany between the molecular disciplines and those that concentrate on the behavior of whole organisms. Many of these interactions with the external world must take place through the microbial cell surface.

Modern studies of bacterial cell surfaces began half a century ago, with the isolation of cell walls by M. R. J. Salton and others in 1951, the discovery of what later turned out to be the precursors of cell wall peptidoglycan by J. T. Park and M. J. Johnson in 1949, and the isolation of pure lipopolysaccharides (LPS) by O. Westphal and O. Lüderitz in 1952 (see reference 45). All these research areas soon went through explosive development, so that by the mid-1960s the major structural features of peptidoglycan, teichoic acids, and LPS were already elucidated. However, these studies may have seemed too "static" for a few scientists. S. E. Luria and H. M. Kalckar, who were then interested in the interactions of bacteria with colicins and phages, respectively, thus organized a series of little meetings beginning in 1961, on a subject that Kalckar would later call "ektobiology" as a pun of sorts on the talk of exobiology that was fashionable then, during the period of competitive launching of Soviet and American space satellites (20). Luria called the field "microdermatology," jokingly claiming that he wanted to grow hair on bacteria, pointing to his balding scalp. Obviously, the center of interest was the roles that cell surface structures played in the "social" behavior of cells, an interest that was stimulated by the then-emerging notion that interaction at the cell surface was crucial in controlling the growth behavior of animal cells (20).

The author was invited to the first of these meetings thanks to a paper with T. Fukasawa (12) that showed that *galE* mutants of salmonella, deficient in galactose synthesis, showed defects in LPS synthesis, a conditional defect that could be rescued by adding galactose to the growth media. These changes obviously altered the social behavior of the cells, with the mutant that was resistant to phage P22 becoming fully sensitive after growth in galactose-containing media, because P22 uses the O-side-chain portion of LPS as the receptor. For a young scientist who was doing these studies without any sense of perspective, the meeting was an eye opener, and I have stayed in the area of bacterial cell surfaces ever since, joining Kalckar's laboratory at Massachusetts General Hospital in 1962. Kalckar's hypothesis was that cell surface glycans (often containing galactose) must be involved in cellular recognition processes in both microbial and animal cells (20). In a way, our study of *galE* mutants was a negative picture of such a phenomenon, because wild-type cells escape nonspecific phagocytosis thanks to the hydrophilic sugar chains of LPS, whereas the mutants are avirulent because they produce drastically truncated LPS (12).

Structure and biosynthesis of cell surface glycans. We can now see the 1960s as the period in which we acquired much of our basic knowledge on cell wall peptidoglycan and LPS. Studies on peptidoglycan biosynthesis, begun in 1949 with the isolation of the "Park nucleotide" from penicillin-treated bacterial cells, were developed beautifully, most prominently by J. L. Strominger (47), first through the identification of the nucleotide as UDP-*N*-acetylmuramyl-pentapeptide and the realization that it represented starting material for peptidoglycan synthesis and then with the careful characterization of each of the enzymatic steps. Importantly, these studies were complemented by the structural studies carried out by degrading peptidoglycan with enzymes of different specificities (13).

Similarly, the biosynthetic studies on LPS carried out in the laboratories of M. J. Osborn (39), P. W. Robbins (44), and myself (33) were complemented and sometimes even guided by structural data from the laboratory of Lüderitz and Westphal (27). Furthermore, because the peripheral part of LPS is not necessary for bacteria growing as pure cultures in the laboratory, mutants defective in LPS biosynthesis could be isolated and were an invaluable help (28).

When these studies were initiated, practically nothing was known about the biosynthesis of complex polysaccharides. Indeed, it was even suggested that the structure of the product may be determined by a template mechanism that utilized the different nucleotide "handles" for various sugars (CDP-abequose, TDP-rhamnose, GDP-mannose, etc. in the biosynthesis of *Salmonella typhimurium* LPS). In comparison with our ignorance at the beginning, what we learned in the latter half of 1960s was impressive. It was established that the "core" of LPS is made by the successive addition of each sugar, the sequence being determined entirely by the specificities of transferases. In contrast, the peripheral O side chain, which consists of many repeats of an oligosaccharide unit, was found to be made first by assembly of the repeating unit on a lipid carrier (49), which was then found to become polymerized. (This discovery was accompanied by the simultaneous observation that a similar lipid carrier also functions for peptidoglycan synthesis [1]). The carrier lipid was soon identified as C_{55}-undecaprenol (50). Later studies suggested that the repeating unit is flipped over to the outer face of the membrane before polymerization (29), and indeed the recent sequencing of the *rfb* gene cluster, responsible for the O-chain synthesis, showed the presence of the *rfbX* gene, which codes for a protein with 12 transmembrane helices that may catalyze this process (18). The polymerized O chain is finally transferred onto the finished LPS core to complete LPS synthesis.

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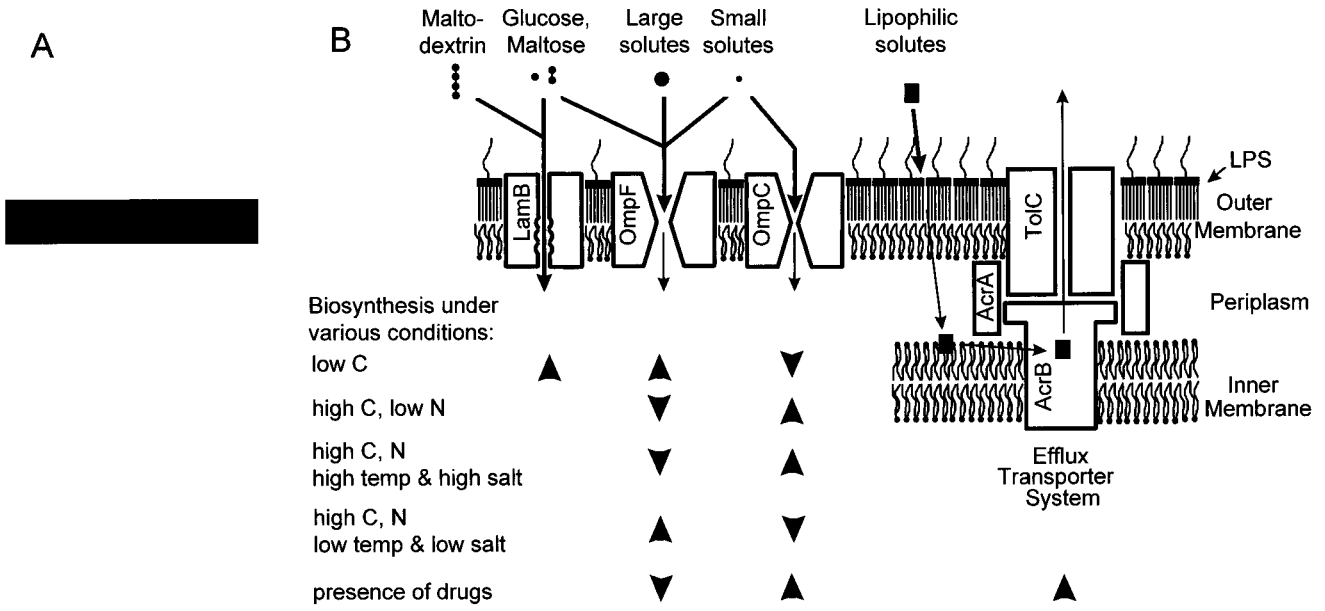

FIG. 1. Structure and barrier functions of the outer membrane of *E. coli* K-12. (A) A typical representation of the outer membrane [then usually called the "lipopolysaccharide-(lipo)protein layer"] in microbiology textbooks in the early 1970s. (B) A current model of the outer membrane. Hydrophilic solutes cross this barrier mainly via channels in nonspecific porins OmpF and OmpC and specific channels such as LamB. Smaller solutes can use both OmpF and OmpC, but larger solutes (including most inhibitors) can only go through OmpF, with its wider channel. In either case, diffusion is slowed down drastically by the narrow opening in the channel, shown by the different widths of arrows. Influx of maltose, maltodextrins, and glucose is facilitated by the LamB channel, with its specific sugar-binding site within the channel. Large, lipophilic solutes traverse the lipid bilayer region of the membrane, but again diffusion is slowed down by the presence of the outer leaflet, which contains only saturated fatty acid residues of LPS. Such lipophilic solutes tend to be pumped out directly into the medium by the multisubunit efflux transporters, such as AcrA-AcrB-TolC (shown here) or EmrA-EmrB-TolC. Although the efflux catalyzed by such a pump may be slow, as indicated by the thin arrow, it will still be effective as it works in synergy with the outer membrane barrier. Regulation of biosynthesis of various transporters under different conditions is shown at the bottom of the figure. Upward-pointing arrowheads indicate increased synthesis, and downward-pointing ones show decreased synthesis. For details, see text.

Structure and functions of the outer membrane. Electron microscopy showed that the "cell wall" of gram-negative bacteria consists of a trilaminar structure resembling a unit membrane, as well as a more electron-dense peptidoglycan layer underneath, which in turn is found outside the cytoplasmic or inner membrane. Accordingly, the term "outer membrane" (emphasizing the double-membrane construction of the gram-negative cell envelope) was already in use in 1964 (2). Once we began to view the LPS-containing structure as a bona fide membrane, its biological functions suggested themselves, because the most fundamental function of any biological membrane is to serve as a barrier that separates the inside from the outside. However, the outer membrane must somehow allow the passage of nutrients and waste products. Since LPS was uniquely present in the outer membrane, by making LPS-phospholipid mixed vesicles we tested our simple-minded hypothesis that LPS makes the outer membrane generally leaky, with completely negative results (37). This led on the one hand to the finding that *S. typhimurium* outer membrane acted as a molecular sieve for hydrophilic solutes, allowing the ready passage of only these compounds of less than roughly 650 Da (32), and on the other hand to systematic searches for outer membrane components producing permeability of this type, culminating in the identification of porins (31).

It was already common knowledge that gram-negative bacteria are more resistant to lipophilic dyes, detergents, and most lipophilic antibiotics than gram-positive bacteria. Now that we knew that the permeability of the outer membrane to hydrophilic solutes could be explained by the presence of porins, we considered the possibility that LPS-containing bilayers were less permeable (contrary to our earlier assumption) to lipophilic compounds than the common phospholipid bilayers. We have shown that at least in enteric bacteria the outer leaflet of the outer membrane contains no detectable amounts of glycerophospholipids (21) and thus by inference contains LPS only (Fig. 1B). L. Leive showed (25) that EDTA treatment of whole cells of *Escherichia coli* removes mostly LPS and at the same time makes the cells hypersensitive to lipophilic agents. I further observed that lipophilic probes such as nafcillin apparently failed to enter wild-type *S. typhimurium* cells (34) and hastily concluded that the LPS-filled outer monolayer made the outer membrane practically impermeable to lipophilic solutes. Many years later, P. Plésiat brought to my laboratory a clone of *Pseudomonas testosteroni* sterol dehydrogenase. Incubating various gram-negative cells containing this clone with steroid hormones allowed us to measure the outer membrane permeability to these probes, which were immediately oxidized by the enzyme the moment they crossed the cell envelope. These experiments showed that the asymmetric, LPS-containing bilayer was indeed a significant barrier, decreasing the penetration rates of these lipophilic molecules to about 1/100 of their penetration rates across the usual phospholipid bilayer membranes, yet the probes did go through the outer membrane with a half-equilibration time of only a few seconds (42). The reason why nafcillin did not enter *S. typhimurium* cells was that it was pumped out by a multidrug efflux pump with an incredibly wide specificity, AcrAB (35, 36) (Fig. 1B).

In the last example above, we see that what originally appeared to be a static permeability barrier was actually the result of an active, dynamic transport process. Bacterial cells can also modulate the seemingly static porin permeability in surprising ways. Most *E. coli* strains produce two porin mole-

cules, OmpF and OmpC, whose channel diameters are thought to differ by only about 10% (38) (Fig. 1B). Nevertheless, because even the larger channel of OmpF is quite narrow (7 by 11 Å [6]), this translates into a very large difference in the diffusion rates of most inhibitors, which are often relatively large molecules. Thus, for *E. coli*, the default pattern would be to express mostly the smaller OmpC channel for self-protection to prevent the entry of inhibitors, unless other requirements become more important. In fact, in its normal habitat, which is rich in inhibitors such as bile salts and free fatty acids, the synthesis of the larger OmpF channel is downregulated by utilizing the high temperature (37°C) and high salt concentration (0.1 to 0.2 M) as signals (43). However, in natural waters, where both temperature and salt concentration are much lower, OmpF porin becomes predominant, allowing the cells to accumulate nutrients more efficiently from the environment (Fig. 1B). We would also expect that OmpF expression should increase when the cells are starved for some nutrients, even at 37°C and in high-salt medium. Indeed, starvation for glucose increases *ompF* transcription about 20-fold (26) (Fig. 1B). In contrast, under glucose excess, NH_3-limited conditions, OmpC becomes essentially the only porin, a reasonable solution for *E. coli* as the small ammonia molecules (or ions) would have no difficulty in diffusing through the smaller OmpC channel (Fig. 1B). Finally, the presence of certain antibiotics increases the synthesis of efflux pump AcrAB, at the same time decreasing the synthesis of large-channel porin OmpF through the action of the MarA global regulator that was discovered by S. B. Levy; this tends to increase the resistance to antibiotics more effectively by slowing down their influx and by accelerating their efflux (reviewed in reference 35) (Fig. 1B).

A few outer membrane porins are specific. LamB (46), the "phage λ receptor protein," not only allows the efficient diffusion of maltodextrins, some of which are too large to diffuse through the regular porin channel, but also facilitates the influx of maltose and glucose. The synthesis of LamB is induced by maltose but also by carbon starvation, and under the latter conditions, LamB becomes the major pathway of glucose entry (8) (Fig. 1B).

Electrophysiological studies show that porin channels open and close under various conditions (9), but we do not know the relevance of these in vitro observations to the physiology of whole cells. This reminds us that there are many areas still waiting for exploration, in spite of the fact that we now know so much about the outer membrane (including the high-resolution structures of pore-forming proteins [6, 46]), in comparison with our ignorance only two decades ago (textbooks in the early 1970s did not mention the outer membrane, let alone its functions [Fig. 1A]).

Interestingly, mycobacteria, which belong to the high-GC, gram-positive bacterial group, were found to have the outer layer of their rather impermeable cell wall organized essentially as a lipid bilayer, with porin(s) to allow the diffusion of hydrophilic solutes (17). Although the less-fluid leaflet of this bilayer is the inner leaflet, in contrast to the gram-negative bacterial outer membrane, in which the outer leaflet is less fluid, the similarity in the construction is striking. Interestingly, these high-GC, gram-positive bacteria appear to be most closely related to the gram-negative bacteria if the sequences of several proteins are used as the criterion (15).

Current perspective. When Luria and Kalckar advocated studies on bacterial cell surfaces almost 40 year ago, their major interest was on the roles surface polymers may play in the cell-to-cell interactions, as mentioned earlier. We have indeed come a long way in this area. Cell-to-cell interaction among bacteria obviously may occur in the community of bacteria growing as biofilms. It has been known that bacteria in biofilms behave differently (for example in being much more resistant to antibiotics) than those in a free-swimming form, but little attention has been paid so far to the consequences of interactions between cells. However, the regulation through the production (and presumably high local concentration) of autoinducers has been established (7). One would expect an even larger role in contact-based or short-range interactions in microorganisms with "social" life styles, such as myxobacteria or slime molds. C signal in *Myxococcus xanthus* is indeed thought to be generated by a surface-located protein and exchanged between tightly packed cells (10, 19), and the O chain of LPS is needed for fruiting body formation (3).

Cell surface glycans obviously play important roles in the interaction of symbiotic or pathogenic bacteria with their host cells. In the classical scenario for pathogens, seen for example with pneumococci, bacterial exopolysaccharides protect pathogens against nonspecific phagocytosis. Because recognition by antibodies will nevertheless result in successful phagocytosis, it is advantageous for pathogenic bacteria to produce glycans for which the host will have difficulties in producing antibodies. These glycans frequently contain unusual components: for example, *Salmonella* LPS often contains rare 3,6-dideoxyhexoses. Some pathogens even go to the length of producing glycans that look like the glycans on host cells, a phenomenon called molecular mimicry (see reference 30). In a remarkable example, some human pathogens not only produce LPS whose structures mimic those of human cell surface glycolipids but also uses their enzymes and host donor compounds to sialylate their LPS, presumably so that their cell surface will look even closer to the host cell surface. With symbionts, in contrast, it would be more advantageous to have the bacteria recognized by host cells. When *Rhizobium* cells interact with the roots of plants, the early stages are dominated by low-molecular-weight compounds, nodulation factors. However, when the bacterial cells reach the epidermis layer through infection threads, then exopolysaccharides on the surface of *Rhizobium* (or oligosaccharides derived from them) become indispensable for bacterial invasion of continually elongating nodules (24). Most interestingly, the invasion defect in *exo* mutants can be rescued by the addition of exopolysaccharides from strains that normally nodulate that particular plant but not those from strains that nodulate other plant species. Thus, the role of exopolysaccharides here is specific.

The first step in bacterial pathogenesis in humans and animals is usually the specific recognition by a bacterial surface component of a specific component of the host cell surface. In the evolution of such a specific recognition process, it is easier to fine-tune the structure of the protein partner than that of the carbohydrate partner, because the structure of the latter can be changed only in large increments. It was proposed (4) to call the active partner (thus usually a protein) a "cognor" and the passive partner (usually carbohydrate) a "cognon." Many gram-positive pathogens recognize and adhere to the components of extracellular matrix of the host, and cognor proteins of bacteria in this case have been called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (40). Adhesion of *E. coli* through Pap pili to the cell surface glycolipids containing α-galactosyl-(1→4)-β-galactose structure, present on the surfaces of cells of some humans (48) is another classical case of a bacterial cognor recognizing a specific cognon on animal cells. Most interestingly, it now appears that this interaction signals changes in the bacterial cell (51), as well as in the host cell (16). In some cases, however, such specific recognition is used by host cells to "clear" infecting organisms: cystic fibrosis transmembrane regulator (CFTR) on

airway epithelial cells recognizes LPS on *Pseudomonas aeruginosa* to initiate clearing, and this explains the common occurrence of *P. aeruginosa* infection in cystic fibrosis patients, who have defective processing of CFTR (41).

Many bacterial pathogens must invade nonphagocytic host cells. Paradigms of such interactions involve the invasion by *Shigella* and *Salmonella* cells of a special class of epithelial cells of the small intestine. This process occurs by the stimulation of host cells, which are excited to produce spectacular changes in the local cytoskeleton network and then to engulf bacterial pathogens in their vacuoles. This stimulation of the host cells was recently found (see reference 11) to be caused by injection of a few bacterial proteins into the host cells through the contact-dependent type III secretion systems, which are distributed widely, not only among animal pathogens but also among plant pathogens (5, 23). Secretion machinery of this type becomes activated by "contact" with the host cell surface, but the factor that creates specificity in this interaction is still largely unknown. A fascinating observation was made: *Salmonella* cell surface assembles, upon contact with epithelial cells, an appendage (14) which apparently is based on a syringe-like apparatus reminiscent of a flagellar basal body, marking the first time the type III secretion apparatus has been visualized (22). Luria, if he were alive today, would be beside himself learning that *Salmonella* cells truly does grow "hair" in a matter of minutes. This is indeed an exciting period for microbiology and especially for the biology of microbial cell surfaces.

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